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PRINCIPAL INVESTIGATOR: Bo Lu, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Vanderbilt University
Nashville, TN 37235-7749

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Bo Lu, M.D., Ph.D.

E-Mail: bo.lu@vanderbilt.edu

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14. ABSTRACT

We proposed to determine whether overexpression of survivin results in radioresistance and the possible mechanisms; whether regulators of survivin serve as targets for radiosensitization. We also found that deregulation of survivin in breast cancer is mediated by Stat3 (Signal transducer and activator of transcription). Co-inhibition of survivin and Stat3 results in significantly increased sensitization of breast cancer. In addition, we found that inhibition of apoptosis induces autophagy in cell culture model of breast cancer. Z-VAD, a pan-caspase inhibitor, resulted in further tumor growth delay in a xenograft model of breast cancer.

15. SUBJECT TERMS
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1. Introduction:

We have proposed to determine whether overexpression of survivin results in radioresistance and the possible effects on cell death mechanisms.

2. Body:

To accomplish the proposed studies: we have the following Statement of Work:

Task 1. To determine whether overexpression of survivin results in radioresistance and the possible mechanisms (Months 1-9):

We have shown survivin overexpression leads to radiation resistance as demonstrated in reference 1.

Task 2. To determine whether irradiation downregulates p34CDC2 and its mechanism in vascular endothelium and whether CDK inhibitors sensitize it to radiation injury. (Months 9-19):

Task 3. To determine the mechanism of survivin deregulation in breast cancer and whether inhibition of survivin or its regulator, p34CDC2 abolishes radioresistance. (Months 20-28):

We found that deregulation of survivin in breast cancer is mediated by Stat3. Inhibition of apoptosis induced autophagy, which surprisingly led to radiosensitization.

Task 4. To determine the biological significance of combining survivin inhibitors or CDK inhibitors with radiotherapy in xenograft models of breast cancer (Months 29-36)

We found that inhibition of caspases by Z-VAD led to radiosensitization in both cell culture and xenograft models of breast cancer.

3. Key Research Accomplishments:

1. Survivin overexpression leads to radioresistance.
2. Stat3 mediates deregulation of survivin in breast cancer.
3. Inhibition of apoptosis by caspase inhibitor results in radiosensitization both in vitro and in vivo.

4. Reportable outcomes:

1. Kwang Woon Kim and Bo Lu. Inhibition of caspases upregulates autophagy, sensitizing breast cancer cells to irradiation (in review, Oncogene, 2007).

Abstract: Radiation therapy is an important component of breast cancer treatment, which remains the second leading cause of cancer death amongst women. Autophagy is an alternative cell death pathway previously found to be upregulated when apoptotic machinery is defective. The role of autophagy in cancer therapy remains poorly understood. We therefore investigated whether inhibition of caspases might enhance the cytotoxic effects of irradiation on MDA-MD-231 breast cancer cells. We found that N-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone (Z-VAD), a pan-caspase inhibitor, markedly radiosensitized breast cancer cells both in vitro and in mice. This therapeutic benefit was well tolerated in our mouse model. The enhanced radiosenstization was associated with an upregulation of pro-autophagic proteins ATG5 and Beclin-1, and an increase in characteristic punctate green fluorescent protein-tagged light-chain 3 staining. This novel treatment strategy resulted in an inhibition of tubule formation in endothelial cells, as well as a synergistic decrease in angiogenesis activity in mouse tumor sections. These findings were observed in the context of decreased apoptosis

and less cellular proliferation as assessed by TUNEL and Ki67 staining. This is the first report demonstrating *in vivo* that induction of autophagic cell death through inhibition of pro-apoptotic proteins radiosenstizes cancer cells. This strategy may be of therapeutic benefit for cancer patients.

1. Caspase Inhibitor Z-VAD radiosensitizes MDA-MB-231 breast cancer cells.

To investigate whether inhibition of apoptosis would result in radiosensitization of MDA-MB-231 breast cancer cells, we used clonogenic assay to examine the effects of Z-VAD, an inhibitor of caspases. MDA-MB-231 breast cancer cells were treated with Z-VAD or DMSO control, and were then irradiated with 0-6 Gy. Surviving colonies were counted 8 days later and graphed as survival curves (Figure 1). The radiation dose enhancement ratio (DER) was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus Z-VAD treatment for a surviving fraction of 0.25. Enhanced radiosensitization was demonstrated in breast cancer cells treated with Z-VAD, with a DER of 1.31 ($p<0.003$). As is commonly seen with DMSO treatment which is known to possess some toxicity, there was a small decrease in surviving fraction of DMSO treated cells at high doses of irradiation.

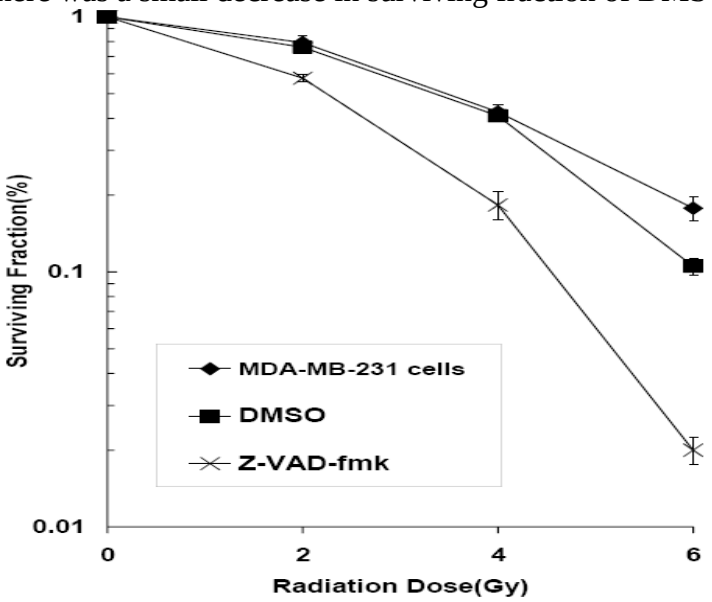


Figure 1. Radiation sensitization of breast cancer cells following inhibition of caspases by Z-VAD with induction of pro-autophagic signaling.
Clonogenic assay revealing radiosenstization of MDA-MB-2331 treated with pan-caspase inhibitor Z-VAD. MDA-MB-231 cells were treated with Z-VAD or DMSO control, and were irradiated with the indicated doses of radiation. After 8 days, colonies were stained and scored. Shown are the mean +/- the standard deviation of three separate repeated experiments.

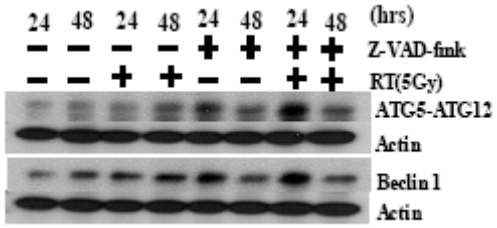
2. Radiosensitization of breast cancer cells is associated with an induction of autophagic proteins and autophagasome formation.

We next sought out to establish whether autophagy was upregulated in response to combination therapy of Z-VAD and irradiation which resulted in effective killing of breast cancer cells. We used Western Immunoblot to examine the expression of the pro-autophagic proteins Beclin-1 and the ATG5-ATG12 complex. MDA-MB-231 breast cancer cells were treated with Z-VAD, and protein extracts from the cells were made at 0, 24 hours, and 48 hours following 5Gy of irradiation (Figure 2A). Treatment with irradiation or Z-VAD alone resulted in an increase in expression of autophagic proteins with a greater amount of ATG5-ATG12 and Beclin-1 seen with the apoptotic inhibitor. The greatest induction was seen following combination treatment at 24h. This effect was less pronounced 48h following treatment. These results suggest that radiosensitization subsequent to treatment with Z-VAD is associated with up-regulation of autophagy.

To confirm this association we examined the effects of caspase inhibition on autopagy by transfecting MDA-MB-231 breast cancer cells with green fluorescent protein-tagged light-chain 3 (GFP-LC3) plasmid (10). Microtubule associated protein-1 LC3 is an important constituent of mammalian autophagosomes, and GFP-LC3 has been demonstrated to be an effective marker of their presence (11). We observed a change in subcellular localization of GFP-LC3 in response to treatment with Z-VAD and

irradiation, from one of diffuse scattering throughout the cytosol to a punctate pattern characteristic of autophagosome formation. Quantitative analysis of this effect revealed very little increase in transfected cells displaying the signature punctate fluorescence pattern of autophagy following irradiation treatment alone at 24 hours (Figure 2B). There were more punctate GFP cells following treatment with Z-VAD in comparison to irradiation, with just over 15% of cells displaying the characteristic pattern of autophagy. When gamma radiation and Z-VAD treatments were combined, however, there was a synergistic, greater than 40% increase in the number of punctate GFP cells.

A).



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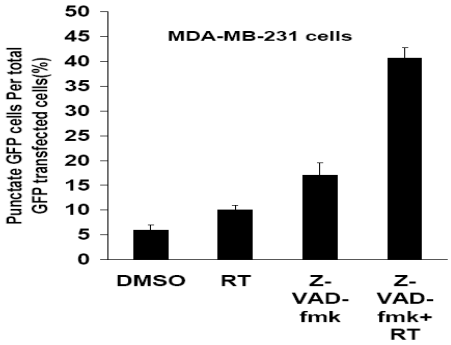
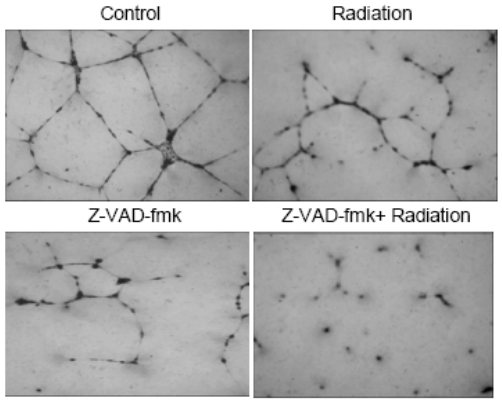


Figure 2. Induction of autophagic proteins and autophagosome formation with Z-VAD and irradiation therapy
A, MDA-MB-231 cells were treated irradiation, Z-VAD, or a combination of both. Cells were collected at noted time points and protein extracts were made for Western Immunoblotting. Shown are immunoblots of ATG5-ATG12 complex and Beclin 1 using lysates from breast cancer cells. B, GFP-LC3 transfected cells were treated with 5Gy, Z-VAD, or both and then examined by fluorescence microscopy after 24 hours. The percentage of cells with punctate GFP-LC3 fluorescnece was calculated relative to all GFP-positive cells. Error bars are shown as mean ± S.D.

3. Z-VAD decreases angiogenesis in irradiated human umbilical vein endothelial cells (HUVECs)

We next investigated what effect inhibition of caspases would have in combination with irradiation on angiogenesis. We treated HUVECs with Z-VAD and then 5Gy irradiation, stained the cells and examined their differentiation into capillary-like tube structures under the microscope. The average number of tubes for three separate microscopic fields (100x) and representative photographs were taken. Treatment with Z-VAD irradiation alone significantly decreased angiogenesis (Figure 3A,B). The most dramatic inhibition of microtubule formation, however, was again seen following combination treatment with Z-VAD and irradiation, with greater than a five-fold reduction in tubule formation in comparison to irradiation therapy alone.

A).



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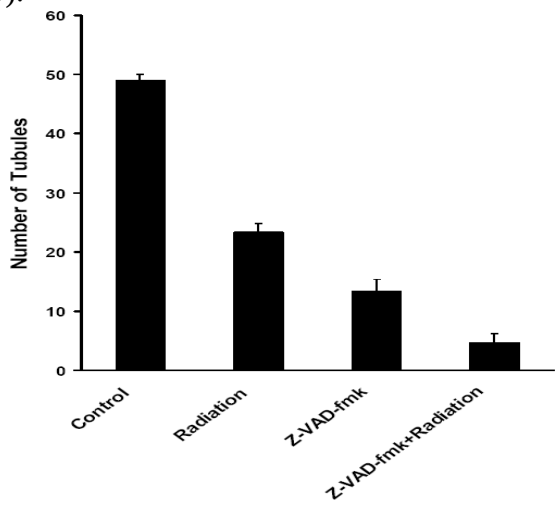
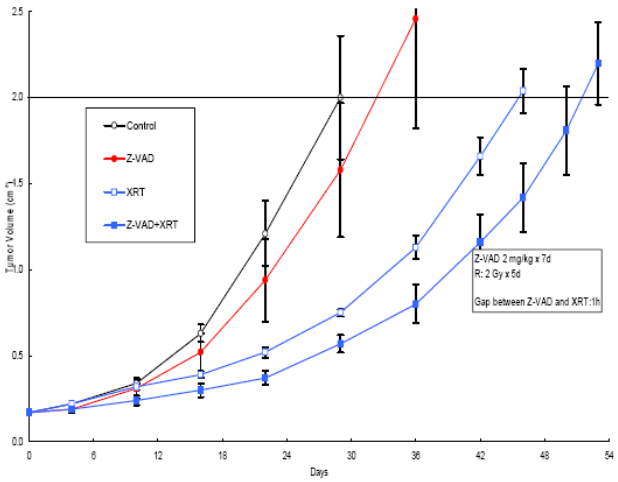


FIGURE 3. INCREASED ANGIOGENESIS AFTER Z-VAD AND IRRADIATION TREATMENT.
A,B, HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS) WERE TREATED WITH Z-VAD, 3GY, OR COMBINATION THERAPY. A, REPRESENTATIVE HUVECS AT VARYING LEVELS OF DIFFERENTIATION UNDER MICROSCOPE, H&E STAIN; B, THE AVERAGE NUMBER OF TUBES FOR THREE SEPARATE MICROSCOPIC FIELDS (100X) IS SHOWN ON THE Y-AXIS.

4. Combination treatment of Z-VAD with irradiation results in decreased tumor volume and is well tolerated in an in vivo mouse model

Here and in prior studies on breast and other cancer cell lines we established the effectiveness of this new therapeutic strategy of inhibiting apoptotic machinery and promoting radiosensitization through an induction of autophagic cell death. We therefore next wished to examine the efficacy of using Z-VAD as a radiosensitizing agent in a mouse model to better assess the potential of this technique for therapy in humans. Breast cancer cells were injected subcutaneously in mice and grown for approximately 7 days to an average volume of 0.28cm^3 prior to therapy. The treatment groups consisted of a vehicle control, Z-VAD, vehicle plus radiation, and combination Z-VAD plus radiation. Vehicle control and Z-VAD were administered at doses of 2 mg/kg i.p. for 7 consecutive days. The mice in radiation groups were irradiated 1 hour after Z-VAD treatment with 2 Gy daily over 5 consecutive days. Growth delay was calculated for treatment groups relative to control tumors. The tumor growth curve was prolonged following treatment with Z-VAD and more so when mice were treated with irradiation alone. The greatest prolongation was seen with combination therapy of Z-VAD and irradiation (Figure 4A). Combination treatment was also very well tolerated, with minimal weight loss at ten days in the combined Z-VAD and irradiation group relative to control (Figure 4B). The increase in body weight after this time point in all groups reflected the increase in tumor volume, with combination treatment resulting in the least tumor growth.

A).



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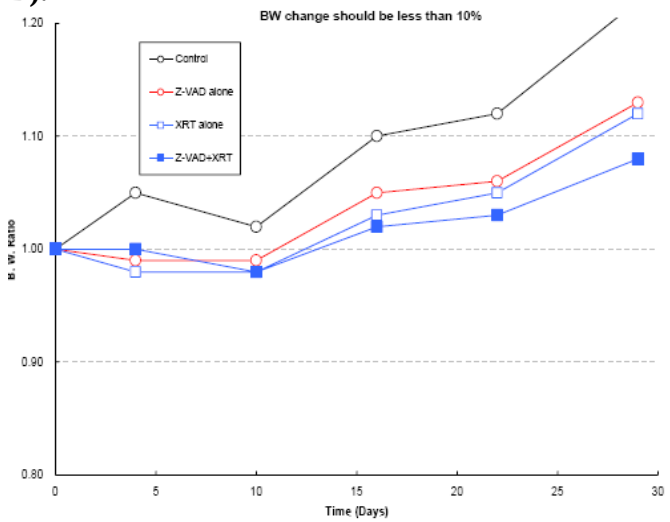


C).

Figure 4. Combination therapy slows tumor growth and is well tolerated.
Human MDA-MB-231 cells were injected into female athymic nude mice and grown for 6-8 days to an average tumor volume of 0.28 cm^3. Treatment groups consisted of vehicle control (in DMSO), Z-VAD, vehicle plus radiation, and Z-VAD plus radiation. A, Tumors were measured regularly and growth delay was calculated for treatment groups relative to control tumors. B, Body weights were measured every 5 days and body weight ratio was calculated relative to baseline measurement.

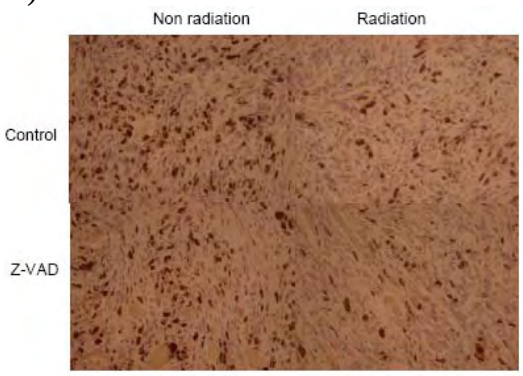
5. Histologic sectioning demonstrates decreased Ki67, vWF, and TUNEL

To further assess the intriguing efficacy of apoptosis inhibition with the pan-caspase inhibitor Z-VAD to radiosensitize breast cancer, we examined fixed tumor sections from implanted mice for ki67, vWF, and TUNEL staining. The four treatment groups included a control, radiation, Z-VAD, and Z-VAD plus irradiation group. Ki67 immunohistochemical staining revealed less cellular proliferation in the irradiated group in comparison to the Z-VAD treated group. Combination treatment yielded the least number of Ki67 stained cells.(Figure 5A, B)

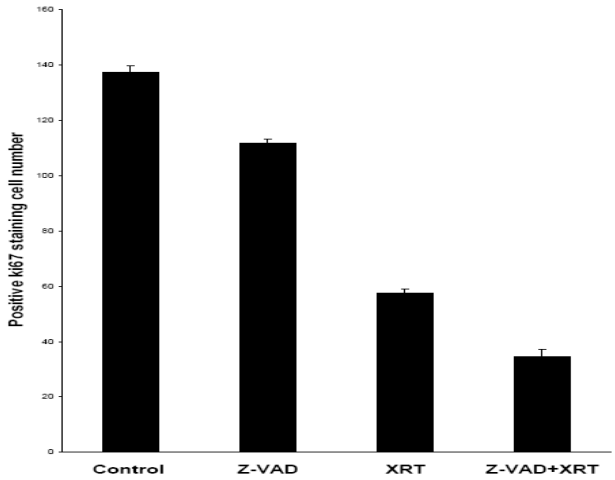
We used von Willebrand Factor (vWF) staining in order to study angiogenesis in MDA-MB-231 breast cancer tumors from sacrificed mice. Similar to what was observed in HUVECs, combination therapy of Z-VAD and irradiation resulted in a 5-fold reduction in the number of staining vessels in comparison to control, and over a 2-fold reduction relative to radiation therapy alone (Figure 5C, D). Representative histological photographs of Ki67 and vWF staining are shown.

The decreased proliferation and angiogenesis was not associated with an increase in apoptosis in the breast cancer tumors as demonstrated by TUNEL staining. Terminal Transferase and Biotin-16-dUTP detection (the TUNEL-Fluorescence Method) detects and quantifies apoptosis at the cellular level by labeling free 3'-OH terminals that result from cleavage of genomic DNA during apoptosis (12). There was 5 times less TUNEL staining cells in Z-VAD treated tumors in comparison to those tumors treated with irradiation (Figure 5E). Combination therapy of Z-VAD and irradiation resulted in nearly four times less TUNEL staining in comparison to the irradiation group.

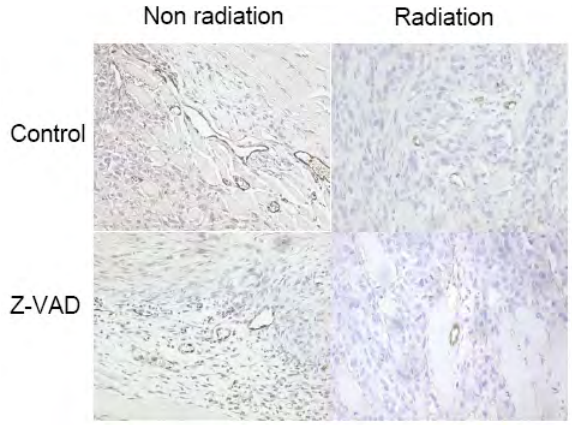
A).



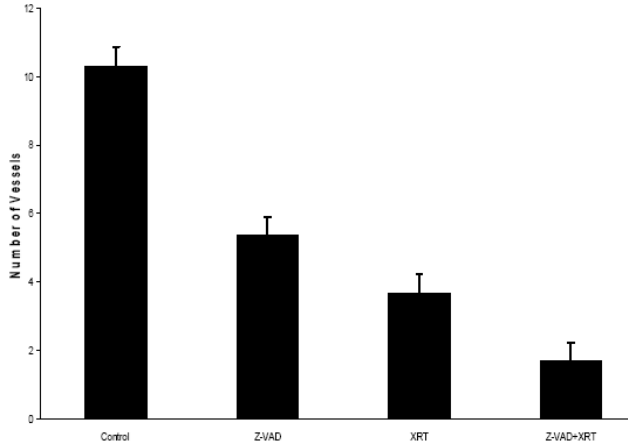
B).


Positive ki67 staining cell number
160
140
120
100
80
60
40
20
0
Control
Z-VAD
XRT
Z-VAD+XRT

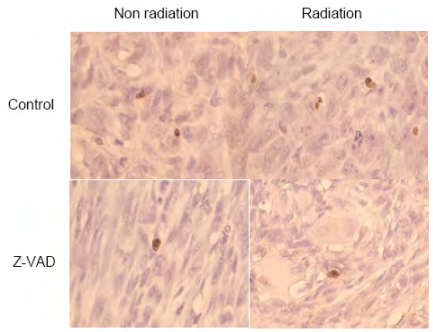
C).


Non radiation
Radiation
Control
Z-VAD

D).


Number of Vessels
12
10
8
6
4
2
0
Control
Z-VAD
XRT
Z-VAD+XRT

E).


Non radiation
Radiation
Control
Z-VAD

F).

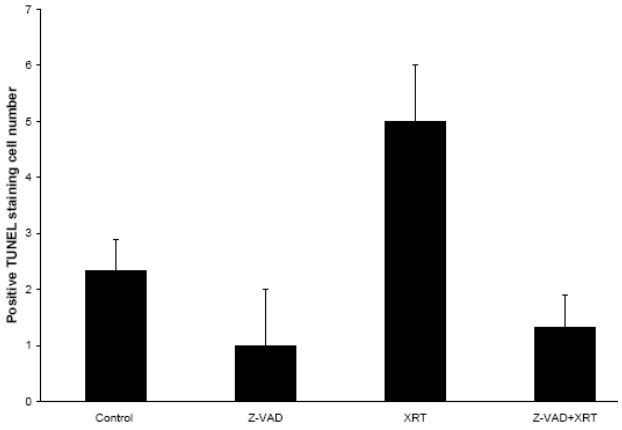


Figure 5. Ki67, vWF, and TUNEL immunohistochemistry staining reveals decreased proliferation and angiogenesis in the absence of up-regulated apoptosis.
Mice were treated and sacrificed and tumors were paraffin fixed. Slides from each treatment group were then analyzed following Ki67, vWF, and TUNEL staining. A, Representative histological section following Ki67 staining. B, Average number of Ki67 stained cells per high powered field. C, Representative histological section following vWF staining. D, Average number of vWF stained blood vessels per high powered field. E, Representative histological section following TUNEL staining. F, Average number of positive TUNEL stained cells per high powered field.

5.Conclusions: We have found that deregulation of survivin in breast cancer is mediated by Stat3. This could lead to radioresistance. Inhibitors of survivin, Stat3, mTOR, and caspases enhance therapeutic effects of radiation. We are currently investigating whether small molecules of caspase inhibitors can sensitize breast cancer in vitro and in vivo. Clinical studies using these compounds will be planned if confirmed by the ongoing preclinical testing.

6. Recent Publications that acknowledged this funding:

1). Luigi Moretti, Kwang Woon Kim, and **Bo Lu**. Crosstalk between Bak/Bax and mTOR signaling regulates radiation-induced Autophagy. Autophagy 2007 Apr-Jun;3(2):142-4.
2). Luigi Moretti, Yong Cha, Kenneth Niermann, and **Bo Lu**. Switch between Apoptosis and Autophagy: Radiation induced-Endoplasmic Reticulum Stress? Cell Cycle. 2007 Apr;6(7):793-8.
3). Jeffrey Albert, Carolyn Cao, Alan Sandler, David Johnson, Jennifer Low, Mace Rothenberg and **Bo Lu**. Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. Clin Cancer Res 2007 13: 3033-3042.
4). Kwang Woon Kim, Robert W. Mutter, and **Bo Lu.** Inhibition of survivin and Aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. Int J Radiat Oncol Biol Phys 2007, 67 : 1519-1525.
5). Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, **Lu B**. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. Cancer Res. 2006 Oct 15;66(20):10040-7.
6). Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and **Bo Lu.** Autophagy for cancer therapy through inhibition of proapoptotic proteins and mTOR signaling J Biol Chem. 2006 Dec 1;281(48):36883-90.
7). Kwang Woon Kim and **Bo Lu**. Stat3 mediates transcriptional downregulation of survivin following irradiation. Molecular Cancer Therapeutics 5(11): 2659-2665, 2006.
8). Carolyn Cao, Jeffrey Albert, Alan Sandler, David Johnson, and **Bo Lu**. Vascular Endothelial Growth Factor Tyrosine Kinase Inhibitor AZD2171 and Fractionated Radiotherapy in Mouse Models of Lung Cancer. Cancer Res 2006 66: 11409-11415.
9). Jeffrey M. Albert, Carolyn Cao, Ling Geng, Lauren Leavitt, Dennis E. Hallahan and **Bo Lu**. Integrin $\alpha_v\beta_3$ antagonist Cilengitide enhances efficacy of radiotherapy in endothelial cell and non-small cell lung cancer models. Int J Radiat Oncol Biol Phys 2006, 65:1536-1543.
10). Jeffrey M. Albert, Kwang Woon Kim, Carolyn Cao, and **Bo Lu.** Targeting the Akt/mammalian target of rapamycin pathway for radiosensitization of breast cancer. Mol Cancer Ther 2006 5: 1183-1189.
11). Carolyn Cao, Eric T. Shinohara, Ty K. Subhawong, Ling Geng, Kwang Woon Kim, Jeffrey M. Albert, Dennis E. Hallahan, and **Bo Lu** Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2 Mol Cancer Ther 2006 5: 411-417
12). Carolyn Cao, Kenneth Niermann and **Bo Lu**. Radiation sensitization of lung cancer and its angiogenesis through inhibition of Clusterin. Int J Radiat Oncol Biol Phys 2005 63:1228-1236.